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Selection and Maintenance of Sexual Identity in the Drosophila Germline

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ABSTRACT

Unlike sex determination in the soma, which is an autonomous process, sex determination in the germline of Drosophila has both inductive and autonomous components. In this paper, we examined how sexual identity is selected and maintained in the Drosophila germline. We show that female-specific expression of genes in the germline is dependent on a somatic signaling pathway. This signaling pathway requires the sex-non-specific *transformer 2* gene but, surprisingly, does not appear to require the sex-specific genes, *transformer* and *doublesex*. Moreover, in contrast to the soma where pathway initiation and maintenance are independent processes, the somatic signaling pathway appears to function continuously from embryogenesis to the larval stages to select and sustain female germline identity. We also show that the primary target for the somatic signaling pathway in germ cells can not be the *Sex-lethal* gene.

SEX determination in somatic cells of *Drosophila melanogaster* is dependent upon an autonomous system that functions transiently in the early embryo (SANCHEZ and NOTHIGER 1983; CLINE 1984). This system measures the relative number of *X* chromosomes to autosomes (the *X*/A ratio) in each nucleus and sets the sexual pathway by controlling the transcriptional activity of a special embryonic promoter, *Sxl-Pe*, of the master regulatory gene, *Sex-lethal* (*Sxl*) (KEYES *et al.* 1992). *Sxl-Pe* is turned on by the signaling system in female (2*X*/2A) embryos, while it remains off in male (1*X*/2A) embryos. The proteins from the *Sxl-Pe* mRNAs set in motion an autoregulatory feedback loop that serves to maintain the female-determined state during the remainder of development. In this feedback loop Sxl proteins promote their own expression by directing the productive female-specific splicing of transcripts expressed from the late or maintenance *Sxl* promoter, *Sxl-Pm*. In males, the maintenance mechanism also operates at the level of RNA splicing. In the absence of the embryonic proteins, transcripts from *Sxl-Pm* are spliced to include a male-specific exon that contains in frame translation stop signals that prematurely truncate the open reading frame. The continued splicing of *Sxl-Pm* transcripts in the nonproductive default pattern during the remainder of the life cycle ensures that the male-determined state is remembered.

Sxl directs subsequent somatic sexual development by controlling several subordinate pathways. These include the dosage compensation system and the somatic sexual differentiation pathway. In the former case, the dosage compensation system is turned off by *Sxl* in females, while it is on by default in males (LUCCHESI and MANNING 1987; GORMAN *et al.* 1993). In the latter case, *Sxl* promotes female differentiation by directing the female-specific splicing of transcripts from *transformer* (*tra*) (MCKEOWN *et al.* 1987). This produces tra protein, which, together with the constitutively expressed cofactor, transformer 2 (tra2) protein, activates the splicing of *doublesex* (*dsx*) transcripts into the female mode (NAGOSHI *et al.* 1988). The female dsx protein produced from these transcripts then executes female differentiation. In males, the *Sxl-tra-dsx* splicing cascade is in the default mode and the dsx protein produced by the default mRNAs directs male differentiation.

While the key steps in somatic sexual development are now well understood, the mechanisms of pathway initiation, memory and differentiation in the sexual development of the germline remain largely obscure. What is clear is that germline sexual development is likely to be quite different from the soma. First, many of the known components of the *X*/A signaling system do not seem to play any role in the germline (SCHÜPBACH 1985; GRANADINO *et al.* 1993; STEINMANN-ZWICKY 1993). Second, the target of the *X*/A counting system, *Sxl-Pe*, is not active in the pole cells of female embryos when it is on in somatic nuclei nor does the promoter appear to function in germ cells at later stages of development (KEYES *et al.* 1992). Third, *Sxl* has been shown to be required for normal oogenesis (SCHÜPBACH 1985) and it has generally been assumed that *Sxl* functions as a master regulatory switch in germline sex-determination, much like it does in the soma (NOTHIGER *et al.* 1989; STEINMANN-ZWICKY *et al.* 1989; GRANADINO *et al.*

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1993; STEINMANN-ZWICKY 1993). However, from our previous studies (BOPP *et al.* 1993), it is conceivable that *Sxl* is required for some aspect of germline development other than establishing and maintaining sexual identity. Fourth, genes downstream from *Sxl* in the sexual differentiation pathway—*tra, tra2* and *dsx*—are not required in female germ cells but rather must function in the surrounding somatic tissue (MARSH and WIESCHAUS 1978; SCHÜPBACH 1982). Finally, pole cell transplantation experiments indicate that the choice of female identity in the germline is not strictly autonomous as it is in the soma. STEINMANN-ZWICKY *et al.* (1989) showed that *XX* pole cells develop along the male mode when transplanted into a male somatic background. By contrast, *XY* or *XO* pole cells appear to have autonomous information for male development and develop in the male mode irrespective of the somatic environment. If one assumes that the default state of germline development is male (*cf.* GRANADINO *et al.* 1993) this would imply that the female pathway requires both an inductive signal from the soma and an autonomous signal dependent upon the *X*/A ratio.

The studies reported here have attempted to gain further insights into the mechanisms that might be involved in initiating and maintaining sexual identity in the germline. For this purpose we have used two molecular markers to determine which sexual pathway the germline is following. One of these is the sex-specific splicing of *Sxl* transcripts and the expression of Sxl protein. The second is the sex-specific expression of the germline-specific gene, *orb* (LANTZ *et al.* 1992, 1994). We describe the expression pattern of these two genes in genetic backgrounds that alter the development of the female germline. Our results indicate that *Sxl* is unlikely to be the master switch in germline sex determination. Instead, our studies suggest a different mechanism for initiating and maintaining germline sexual identity and implicate players in this process that have yet to be identified.

MATERIALS AND METHODS

Fly strains: Flies were maintained on standard yeast/cornmeal medium and kept at 25° unless otherwise indicated. Mutants are described by LINDSLEY and ZIMM (1992). The *dsx* allele is dsx^1, the dsx^{Df} allele is the dsx^{43} allele. The tra^1 and tra^{Df} stocks were obtained from J. BELOTE, the $tra2^{Df}$ allele (*trix*) from W. MATTOX and the $tra2^{ts2}$ and dsx^D (dsx^{Swe}) allele were from B. OLIVER. Stocks not in our collection were from the Bloomington Stock center.

RT-PCR Analysis of gonadal RNA: RNA was prepared from dissected ovaries or testes as previously described (BOPP *et al.* 1993). The sample was treated with acid phenol to reduce the amount of contaminating genomic DNA. Analysis of *Sxl* RNA was as previously described (BOPP *et al.* 1993); *orb* RNA was analyzed similarly. A primer that hybridizes to common *orb* sequences was used to reverse transcribe (FROHMAN *et al.* 1988) the RNA. 5% of the cDNA mixture was then amplified with either a male-specific primer and the common primer in Figure 1A or with a female-specific primer and the common primer. Since both male and female PCRs use the same reverse transcribed material, the amount of male *vs.* female product reflects the proportions of each type of *orb* RNA within a sample. PCR conditions were: one cycle of 95° for 3 min, 62° for 2 min, 72° for 40 min followed by 30 repeats of the cycle 95° for 45 sec, 62° for 2 min, 72° for 1.5 min. Primer sequences were as follows: reverse transcription primer 5′ CTCCATGTGCATGTGGCATC 3′, common primer 5′ CGAGTTCGAGCGCGTGGAAGC 3′ (5′ GTATCGGCGCTGATGTCGAG 3′ for the products in Figure 1B), male primer 5′ CATGTTGGGAGTCGAGAAGCC 3′, female primer 5′ GAGAGGCGGAAGTGGTGACATC 3′. Detection of products was done by Southern analysis with an *orb* cDNA.

Temperature shift experiments: Eight-hour collections (with the exception of the 18° 12-hr time point, which was a 4-hr collection) of $tra2^{ts2}/tra2$ embryos (*w; bw* $tra2^{ts2}$*/CyO* × *pr cn bw tra2/CyO; B*^{s}*Y*) were maintained at the collection temperature of either 18° or 29°. At various time points in development they were then shifted to 29° or 18°, respectively, and left at the new temperature until they reached adulthood. The gonads of the *XX* pseudomales (non-white, non-Bar eyed, non-Curly) were then dissected and the expression state of *orb* assayed as described above.

Immunocytochemistry: Samples were dissected, fixed, stained and imaged as previously described (BOPP *et al.* 1993). The older embryo were vigorously shaken during the devitillinization step to crack the cuticle and stored in methanol at 4° for several days before staining with antibodies. Antibody incubations and washes for these embryos were extended to at least double the normal time. Fluorescent probes were from Jackson ImmunoResearch Laboratories, Inc.

Quantitation of PCR products: Blots were imaged on a Molecular Dynamics Phosphorimager. Counts of the male and female bands were measured less background values to give an estimate of the ratio of products.

RESULTS

Sexual identity of germ cells in mutants that give rise to tumorous ovaries: Pole cell transplantation experiments in the early eighties (SCHÜPBACH 1982, 1985) showed that female germ cells defective in *Sxl* function do not differentiate properly but instead form tumorous cysts consisting of many small undifferentiated one- and two-cell cysts. This observation gave rise to the idea that female sterile mutations exhibiting a similar tumorous ovary phenotype define a group of loci involved in germline sex determination (OLIVER *et al.* 1988, 1990; PAULI and MAHOWALD 1990; PAULI *et al.* 1993; WEI *et al.* 1994). Support for this hypothesis came from the finding that a constitutive allele of *Sxl* can either completely or partially alleviate the tumorous phenotype of some of these mutants (STEINMANN-ZWICKY 1988; PAULI *et al.* 1993; BAE *et al.* 1994).

To explore the possible role of these loci in germline sex determination, we analyzed their effects on the splicing of *Sxl* mRNA and the expression and subcellular distribution of Sxl protein (BOPP *et al.* 1993; LANTZ *et al.* 1994; see also OLIVER *et al.* 1993). The tumorous ovary mutants could be divided into two classes. In the first, class A, are mutations that, at most, only marginally

perturb the female-specific splicing of *Sxl* RNA. This group includes the tumorous alleles of *fused* (*fu*^{1}), *bag-of-marbles* (*bam*$^{\Delta}$), *ovo*^{D1} (*ovo*^{D1}/+; data not shown), as well as two female sterile alleles of *Sxl* (*Sxl*^{f4} and *Sxl*^{f5}). Some of the mutants in this group show abnormalities in the subcellular distribution of Sxl protein, typified by a persistence of cyptoplasmic Sxl protein as the undifferentiated germline cysts age (see BOPP *et al.* 1993). The cytoplasmic rather than nuclear localization of Sxl protein in these cells most likely results in the very low levels of male-spliced *Sxl* RNAs detected in these mutants. In the second, class B, are two mutations, *otu*^{1} and *snf*^{fs1621}, that severely disrupt the splicing of *Sxl* transcripts and the expression of Sxl protein. We found high levels of male-spliced *Sxl* mRNA in ovaries of both mutants. In addition, though the somatic cells of these mutant ovaries had wild-type levels of Sxl protein, no Sxl protein could be detected in the germ cells (BOPP *et al.* 1993).

In somatic cells, where the activity state of the *Sxl* gene determines sexual identity, turning *Sxl* off or on switches all subordinate pathways from the female to the male mode or vice versa. If *Sxl* has a similar function in germline sex determination, then two predictions can be drawn from these data. First, the tumorous mutants in class A that have only very modest effects on *Sxl* expression should not show significant perturbations in the sex-specific expression of other germline genes. Second, the tumorous mutants in the B class (represented by *otu*^{1} and *snf*^{fs1621}), where germ cells have no detectable Sxl protein, female germline sexual identity should be disrupted.

To test these predictions, we examined the expression pattern of *orb* transcripts in these two classes of tumorous ovary mutants. *orb* is only expressed in the germline and has sexually dimorphic transcripts that appear to be generated by distinct sex-specific promoters (LANTZ *et al.* 1992, 1994; see Figure 1A). As illustrated in Figure 1B for wild-type ovaries and testes, the transcripts produced by the female and male promoters can be detected by RT-PCR using appropriate primers for the sex-specific exons and for the downstream common exons. In ovaries, a 480-bp fragment containing the two female-specific exons and the downstream common exon is amplified using the female-specific primer. Using the male-specific primer, wild-type ovaries sometimes have a band of about 490 bp that is the size expected for the amplification of contaminating *orb* genomic DNA rather than mRNA. This DNA amplification product includes the male-specific exon, the short downstream intron and the common exon. In testes, no PCR products are detected using the female-specific primer (the intervening intron is too large to efficiently amplify genomic DNA) while the male-specific primer gives an RT-PCR product of 330 bp, which contains the male-specific exon and the downstream first common exon.

The RT-PCR products from the mutants reveal that the expectations of the first prediction are met: *orb* transcripts in the class A mutants are expressed exclusively in the female not the male mode. This finding would be consistent with the conclusion that the class A tumorous ovary mutations are defective in some aspect of germline development *other than* sex determination. Moreover, it should be noted that this is true even for the *Sxl* mutations, *Sxl*^{f4} (see Figure 1B) and *Sxl*^{f5}. Thus, the abnormal cystoblast divisions observed in these particular mutants (BOPP *et al.* 1993) can not be attributed to a failure to properly establish female identity in the germline.

While the predicted results were obtained for the class A tumorous ovary mutants, this was not true for the class B mutants. In spite of the fact that *snf*^{fs1621} and *otu*^{1} have no detectable germline Sxl protein, the *orb* transcripts in these mutant ovaries are also *exclusively female* (Figure 1, B and C). This finding indicates that the *Sxl* gene does not control all aspects of germline sexual identity and can not function in the germline as a cell autonomous master switch. BAE *et al.* (1994) have drawn similar conclusions from their studies on the sex-specific expression of a variety of other markers.

Effect of the somatic sex determination genes on germline sexual identity: If *Sxl* is not the master switch in germline sex determination, then the genes and regulatory strategies used in setting and remembering germline sexual identity could be quite different from those in the soma. Indeed, the pole cell transplantation experiments described above have suggested that an important component of germline sex determination is somatic signaling. The obvious candidates for genes involved in generating a somatic signal would be members of the somatic sexual differentiation pathway, *tra*, *tra2* and *dsx*. These genes are known to be required in the soma for normal oogenesis. Moreover, it has been demonstrated that *tra* and *dsx* have an effect on the sexually dimorphic size of the developing gonad as early as the first instar (STEINMANN-ZWICKY 1994a,b). To ascertain if the sexual differentiation pathway is involved in somatic signaling, we asked whether mutations in these three genes affect the sex-specific expression of *Sxl* and *orb* in the germline.

dsx: *dsx* is at the bottom of the sexual differentiation pathway and is expressed in a male- or female-specific form. Null mutations in *dsx* disrupt the sexual differentiation of both sexes and mutant animals develop as intersexes. In addition, there are dominant mutations of *dsx* that constitutively express the male form of the *dsx* protein independently of chromosomal sex. When such a *dsx*^{D} allele is heterozygous with a wild-type allele in an *XX* animal, both the male and female forms of the dsx protein are expressed and this results in intersexual development. When *dsx*^{D} is heterozygous with a defi-

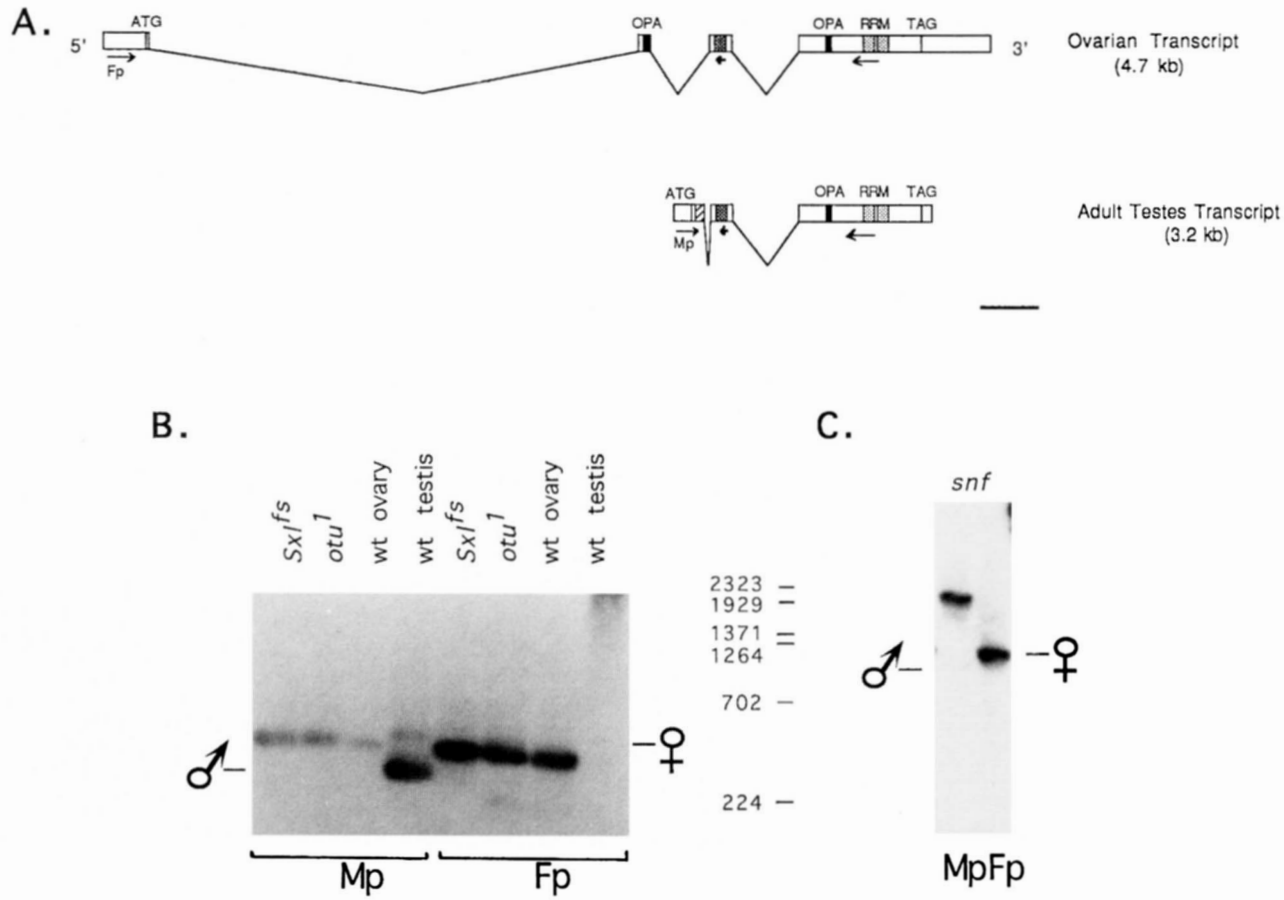

FIGURE 1.—Sex specificity of *orb* RNA in tumorous ovary mutants that affect expression of *Sxl* in the germline. (A) Sex-specific transcripts of *orb*. Rectangles depict exons and the lines connecting the rectangles introns. The positions of primers used in the RT-PCR analyses are shown by arrows below the exons. Fp, Mp show the positions of the female- and male-specific primers, respectively. Arrows that are unlabeled are the primers to common sequences. Horizontal bar represents 1 kb. (B) RT-PCR of *orb* RNA in wild-type (wt) ovaries and testes and in the tumorous ovaries of Sxl^{fs4} (Sxl^{fs}) and otu^{1} homozygous females. The short arrow in A shows the position of the common primer used. This results in a 331 bp-male-specific product and a 483-bp female-specific product. Left 4 lanes show the PCR products when the male-specific primer (Mp) is used, and the right four lanes, when a female-specific primer (Fp) is used. The position of the expected sex-specific product is marked by the appropriate symbol. In testes, the expected product is detected when the male primer is used; no product is detected when a female primer is used. The reverse is true for wild-type ovaries. All ovaries show no male-sized *orb* RNA. Also in all ovary samples is a band above the position of the male product that has the expected size to be derived from genomic DNA [note the small size of the intron between the male primer (Mp) and the first common exon]. (C) RT-PCR of *orb* RNA in snf^{fs1621} homozygous females. Only the female form of *orb* RNA is detected. The long unlabeled arrow in A shows the position of the common primer. This results in a 1027-bp male-specific product and a 1179-bp female-specific product. The band well above the product expected from male RNA is the genomic band (note the larger second male intron). The position of relevant lambda size markers is shown between B and C.

ciency for *dsx*, only the male form of the protein is expressed and *XX* animals develop as males.

If the inducing signal from the soma passes through *dsx*, then at least one of the genotypes—dsx^{D}/Df, dsx^{-}/dsx^{-}, $dsx^{D}/+$—should affect the signal being sent to *XX* germ cells. If the proposed female inducing signal (GRANADINO *et al.* 1993) is dependent upon the female dsx protein (STEINMANN-ZWICKY 1994a,b), this signal would be disrupted in at least two of the three genotypes (dsx^{-}/dsx^{-} and dsx^{D}/Df). We should observe a corresponding alteration in the sex-specific expression of genes in the germline; *Sxl* and *orb* should be in the male not the female mode. In the case of $dsx^{D}/+$, whether an effective female signal would be transmitted to the germline would depend on whether the male form of the dsx protein blocks production of the female signal and/or induces a male signal. (Note that even by other scenarios, if the signal that feminizes or masculinizes the germline is *dsx*-dependent, at least one of these backgrounds should disrupt the signal in *XX* animals and affect the sex-specific expression of *Sxl* and *orb*.)

Shown in Figure 2, A–F, is the pattern of Sxl protein expression in gonads from these *dsx* mutant females, while Figure 2, G and H, shows the RT-PCR analysis of *Sxl* and *orb* transcripts. Morphological analysis of gonads from the different *dsx* mutant combinations reveals ab-

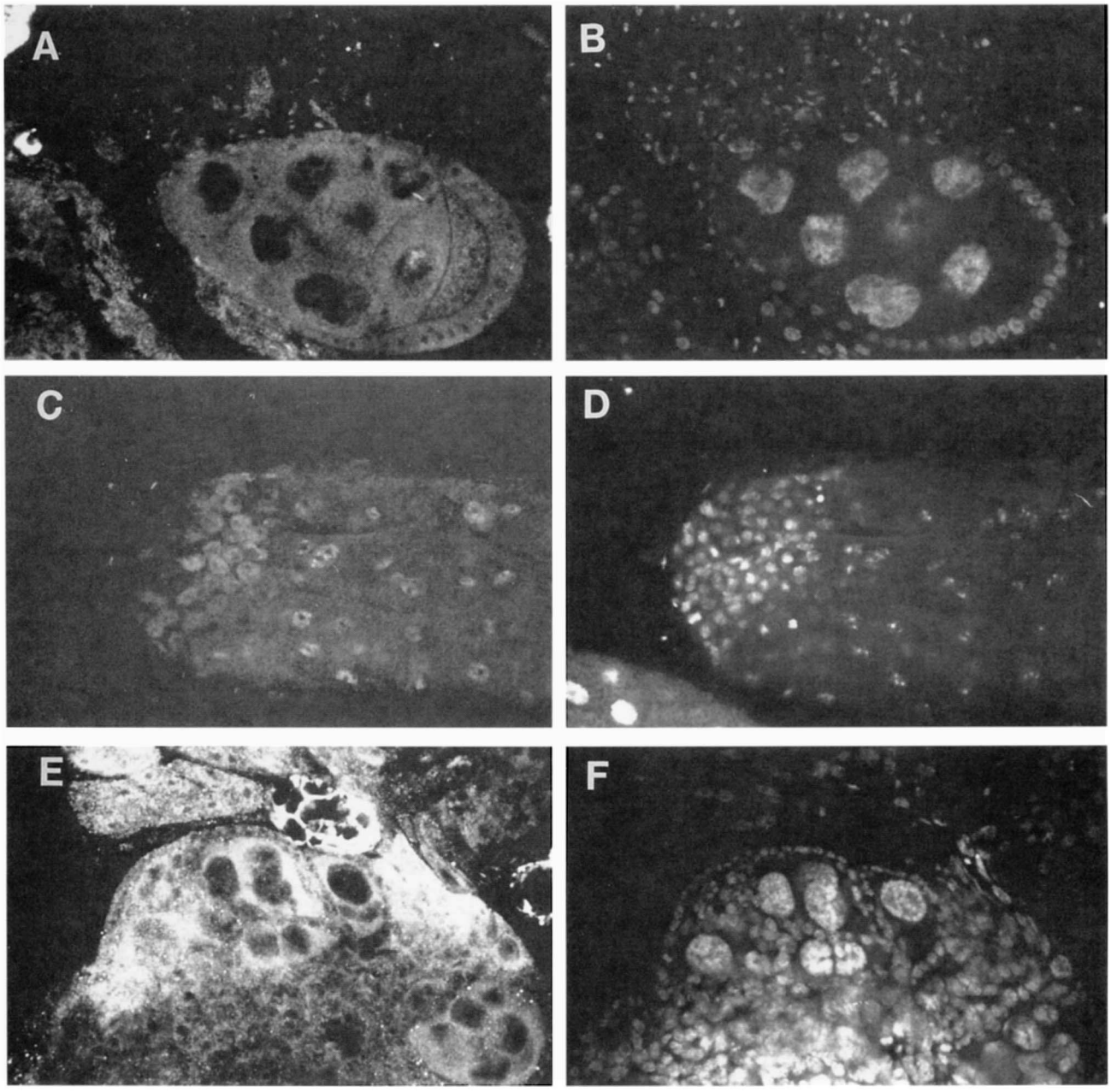

FIGURE 2.—Effect of altering *dsx* activity on the germline expression of *Sxl* and *orb* in females. A, C, and E show Sxl protein, and B, D, and F the corresponding nuclear staining pattern in confocal images of germline tissue. A and B show an egg chamber of around S7 from a dsx^{D}/+ female. The egg chamber appears normal with the exception that the amount of Sxl protein in the "oocyte" appears to be higher than is typically observed in wild-type egg chambers. C and D show the apical end of a pseudotestis from a dsx^{D}/*Df* female. The cells at the very tip of the pseudotestis do not appear to express much Sxl protein. Cytoplasmic Sxl protein is detected where spermatocytes would be located in a normal testis and in cells more distal. E and F show tissue from a dsx^{-}/dsx^{-} female. Cytoplasmic Sxl protein is readily detected in structures that resemble egg chambers. The shape of the chambers and the positioning of follicle cells is abnormal. Large polytenized nuclei resembling nurse cells are present in these chambers (F). Magnification for all images was ×60. (G) *Sxl* RNA splicing in the dissected gonads of dsx^{D}/*Df* and dsx^{-}/dsx^{-} females. A low level of male spliced RNA is detected in both samples (confirmed by probing with the male-specific exon). Expected products for male splicing, 800 bp; female splicing, 610 bp. (H) *orb* expression remains female in dsx^{D}/+, dsx^{D}/*Df* and dsx^{-}/dsx^{-} females. No male *orb* RNA is detected. *orb* RNA is only in the male mode in dsx^{-}/dsx^{-} males (note a doublet is often observed in the male lane even in wild-type males, presumably a PCR artifact). The band above the male products is derived from genomic DNA. Symbols as for Figure 1.

normalities in the development of both the germline and the soma. The least severe defects are observed in dsx^{D}/+ where oogenic differentiation of both germline and somatic cells appears to occur. As illustrated in Figure 2A, egg-chamber-like structures containing polyploid nurse cells, an "oocyte" and surrounding follicle

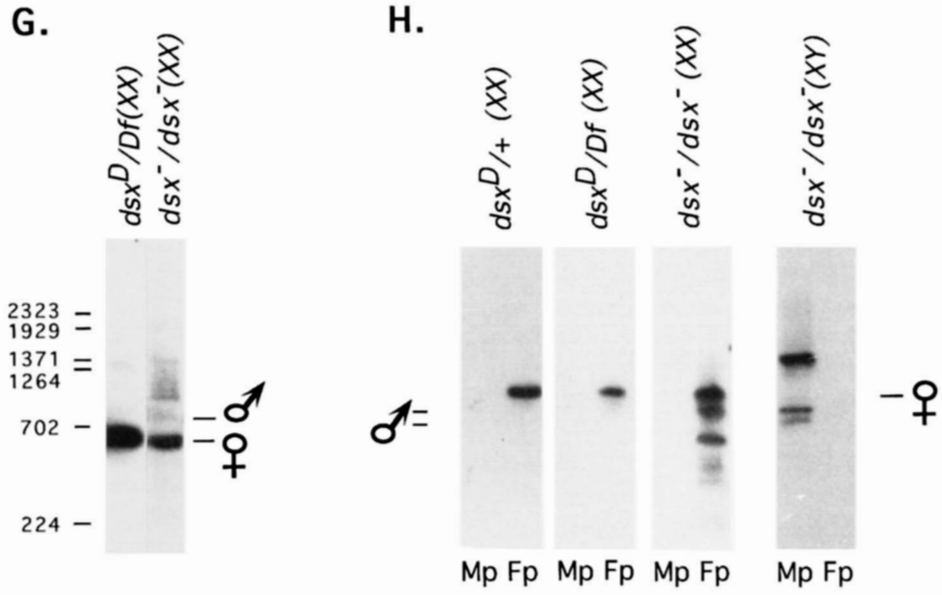

FIGURE 2.—*Continued.*

cells are formed in this mutant background. Sxl protein is clearly expressed in the germ cells. In the chamber shown in Figure 2A Sxl protein is present at high levels not only in the nurse cells but also in the "oocyte." By contrast, in wild-type egg chambers of equivalent developmental stage, high levels of Sxl protein are found in the nurse cells while the oocyte has only very low levels of protein, most of which is in the oocyte nucleus (BOPP *et al.* 1993). In dsx^{-}/dsx^{-} females the germ cells still appear to develop along an oogenic pathway. On the other hand, differentiation of the somatic follicle cells is abnormal; irregularly shaped egg chamber-like structures containing polyploid "nurse cells" are formed, but these "chambers" are not properly surrounded by somatic cells (see Figure 2, E and F). As in $dsx^{D}/+$, Sxl protein can be detected in the germline cells of these gonads. The most severe disruptions in gonadal development are observed in dsx^{D}/Df females. In this genetic background, neither the somatic nor the germ cells appear to follow the oogenic differentiation pathway and the gonad resembles the testis of males. In spite of this failure in oogenic differentiation, Sxl protein is evident in the dsx^{D}/Df germline and, like the class A tumorous ovary mutants described above, the gonads contain small undifferentiated cells with predominantly cytoplasmic protein.

We next used RT-PCR analysis to examine the *Sxl* and *orb* transcripts from these same *dsx* mutants. As expected from the high levels of Sxl protein observed in germ cells of *dsx* mutant gonads, *Sxl* transcripts are spliced predominantly in the female mode in all *dsx* mutant combinations (Figure 2G). Like some of the class A tumorous ovary mutants where Sxl protein remains cytoplasmic, very small amounts of male *Sxl* RNA could be detected in dsx^{-}/dsx^{-} and dsx^{D}/Df mutant gonads. That the germ cells in these different *dsx* mutant combinations are following a female pathway is supported by the RT-PCR analysis of *orb* transcripts (Figure 2H). As might be expected from the oogenic differentiation of the germ cells in $dsx^{D}/+$ and dsx^{-}/dsx^{-} gonads, *orb* RNA is exclusively female in both these backgrounds. *orb* is also exclusively female in the dsx^{D}/Df gonads where, by morphological criteria, the germ cells do not appear to properly execute an oogenic differentiation pathway. These results indicate that *XX* germ cells have been directed to express both *Sxl* and *orb* in the female mode even though the *dsx* mutations cause the surrounding soma to develop inappropriately along an intersexual or male differentiation pathway. Thus, it would appear that *dsx* is not a component of the somatic system that signals "female identity" to the germline (as defined here by the sex-specific expression of these two marker genes).

That the signaling of germline sexual identity can occur independently of *dsx* is further supported by the analysis of *orb* transcripts in *XY* animals that are dsx^{-}. As shown in Figure 2H, *orb* transcripts in dsx^{-} males are expressed exclusively in the male not the female mode.

tra2: The *tra2* gene is expressed constitutively in both sexes and encodes multiple proteins all of which contain an RNA Recognition Motif (RRM) domain. In females the *tra2* gene is required in the soma, but not the germline, while in males it is not required in the soma, but has an essential function in the late stages of spermatogenesis. The best understood function of *tra2* in the female soma is as a co-factor in the *tra*-dependent female-specific splicing of *dsx*. In this regulated splice, tra2 protein provides the sequence specificity, recognizing a repeated sequence motif in the *dsx* female-specific exon. *tra2* also functions as a *tra* co-factor in turning

off a *dsx*-independent differentiation pathway in females that is responsible for male-specific behavior and the formation of the muscle-of-Lawrence (TAYLOR 1992). The likely target gene for *tra* and *tra2* in this pathway is *fruitless* (GAILEY *et al.* 1991). Since *fruitless* does not appear to be required in females, we reasoned that there could be a second *dsx*-independent pathway in the soma that is responsible for signaling the feminization of *Sxl* and *orb* in the germline. Like the behavioral pathway (GAILEY *et al.* 1991; TAYLOR *et al.* 1994), this second *dsx*-independent pathway might require *tra2* as a co-factor to regulate the processing of RNA produced by an unknown downstream target gene ("*Y*" in Figure 8). To test this possibility we analyzed *orb* and *Sxl* expression in various *tra2* mutant backgrounds.

In the first experiment we examined *orb* transcripts in *XX* animals heterozygous for a *tra2* temperature-sensitive allele, $tra2^{ts2}$, and a null allele, *tra2*. At 18°, $tra2^{ts2}$/*tra2 XX* animals differentiate essentially as females but with small or rudimentary ovaries. Consistent with the somatic phenotype, all *orb* RNA is in the female mode at this temperature (Figure 3C). When the temperature is elevated to 25° and 29°, there is a reduction in *tra2* function and *XX* animals develop as pseudomales. This masculinization is accompanied by a change in the pattern of *orb* expression in the germline. At 25° most of the *orb* RNA is expressed in the male mode and only a small amount of female RNA is observed. The switch from female to male is even more complete at 29°, where essentially all *orb* RNA is expressed in the male mode (Figure 3C).

While the loss of *tra2* function at the elevated temperature switches *orb* from the female to the male mode, similar effects were not observed for *Sxl*. Regardless of the somatic phenotype or the temperature at which the flies were raised, Sxl protein could be detected in the germ cells of $tra2^{ts2}$/*tra2 XX* individuals. The *Sxl* antibody staining pattern and morphology of a gonad from a *tra2* pseudomale raised at 25° is shown in Figure 3A. As might be expected from the male pattern of expression of *orb,* neither the soma nor the germ cells show evidence of oogenic differentiation. Instead the gonad resembles a small incompletely developed testis and is populated with many small undifferentiated germ cells. The distribution of Sxl protein in the $tra2^{ts2}$ germ cells is similar to that observed in the class A tumorous ovaries mutants. Relatively high levels of predominantly cytoplasmic Sxl protein are present in the germ cells at the apical end of the $tra2^{ts2}$ gonad, while there is a gradual reduction in protein in the older germ cells in more distal regions of the gonad. PCR analysis confirms that *Sxl* transcripts are spliced predominantly in the female mode with a small amount of male RNA (Figure 3B).

Two hypothesis could explain why the $tra2^{ts2}$ mutation affects *orb* but not *Sxl*. In the first, the sexual state of *orb* and *Sxl* are controlled by entirely independent

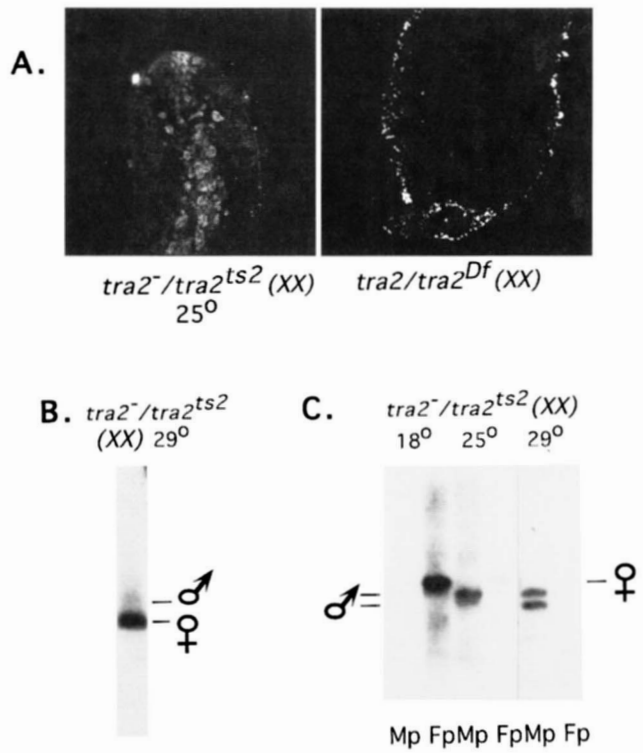

FIGURE 3.—Effect of altering *tra2* activity on the germline expression of *Sxl* and *orb* in females. (A) Confocal image of Sxl protein expression in pseudotestis of the shown genotypes. Cytoplasmic Sxl protein is detected in the germ cells of *tra2*/$tra2^{ts2}$ pseudotestis. When the null allele is placed over a deficiency, only the somatic cells that surround the germ cells appear to express Sxl protein. Magnification was ×60. (B) *Sxl* RNA splicing pattern shows some male RNA. (C) *orb* expression at 18°, 25° and 29° in *tra2*/$tra2^{ts2}$ *XX* animals. At 18° the phenotype is female with small rudimentary ovaries. *orb* is entirely in the female mode. At 25° the phenotype is male and *orb* is predominantly in the male mode. A small amount of female *orb* can be detected. At 29° the phenotype is also male and *orb* appears only in the male mode of expression.

mechanisms. In the second, residual *tra2* activity of the temperature sensitive allele might be sufficient to initiate the *Sxl* autoregulatory loop in the germline at some point in development. Once the feedback loop is initiated, autoregulation would ensure that *Sxl* would be expressed predominantly in the female mode even if the *tra2*-dependent feminizing signal was too weak to efficiently activate the *orb* female promoter.

To distinguish between these two hypotheses we examined *Sxl* expression in *XX* animals carrying the *tra2* null mutation over a deficiency for the locus. These experiments indicate that the second hypothesis is likely to be correct—the sexual state of *both orb* and *Sxl* in the germline is dependent upon *tra2* activity. As illustrated in Figure 3A, this mutant combination affects *Sxl* expression in the germline, and in most cases we detect little or no Sxl protein in germ cells. Infrequently, in the same individual, the germ cells in one gonad have no detectable protein while the germ cells

in the other gonad have very low, but above background levels of Sxl protein. At present it is not clear why we occasionally observe gonads expressing these very low levels of *Sxl* protein. It is possible that this reflects some residual *tra2* activity of maternal origin since we have noticed that if the *tra2* deficiency allele is inherited from the mother, the low levels of Sxl protein are detected much less frequently than if the deficiency allele is received from the father.

tra: The *tra* gene is only active in females and it encodes a 22-D protein with multiple motifs. In the splicing regulation of *dsx,* it appears to provides an Arg/Ser "activation" domain for the tra2 protein bound to the female-specific *dsx* exon (HEDLEY and MANIATIS 1991; HOSHIJIMA *et al.* 1991; RYNER and BAKER 1991). It is likely to function in an analogous manner, using tra2 protein as a sequence-specific co-factor, in the inactivation of the *dsx*-independent male behavioral pathway. Since our analysis of the sexual mode of *Sxl* and *orb* expression implicates *tra2* in the somatic signaling of germline sex, we expected that, like the *dsx* pathway and the *fruitless* behavioral pathway, *tra* would regulate the somatic signaling pathway by providing an "activation" domain to tra2 protein bound to an RNA encoded by a germline signaling gene. If this were the case, null mutations of *tra* should also switch the expression of both *orb* and *Sxl* from the female to male mode in the gonads of *XX* animals.

To test this hypothesis we first examined the expression of *orb* and *Sxl* in the gonads of *XX* animals homozygous for tra^1. tra^1 is a null allele where the *tra* coding sequences are deleted (J. BELOTE, personal communication). To our surprise, we found that both *orb* and *Sxl* are expressed in the female mode in the germ cells of this *tra* mutant. This is illustrated for *orb* by the RT-PCRs shown in Figure 4D. With the female-specific primer, we detect a fragment of the size expected for the amplification product of female *orb* RNA. While some bands are also observed in the male lane, none are of the correct size expected for an RT-PCR product amplified from male mRNA. Essentially equivalent results were obtained with *Sxl* antibody; most of the germ cells in tra^1 mutant gonads express cytoplasmic Sxl protein (not shown, see photo of the tra^1/Df gonad in Figure 4A).

Since these findings were contrary to our expectations, we examined *orb* and *Sxl* in two other genetic backgrounds, tra^1/Df and tra^{v1}/Df, that should also be null for *tra* activity. The deficiency chromosome removes *tra* as well as several surrounding genes, while tra^{v1} has a nonsense mutation at amino acid 13 (J. BELOTE, personal communication). The results for *Sxl* in these two mutant combinations were similar to that for the tra^1 homozygote. As shown for tra^1/Df in Figure 4A, the gonad from *XX* animals deficient in *tra* function contains many small undifferentiated germ cells. Like some of the class A tumorous ovary mutants, these undifferentiated cells have predominantly cytoplasmic Sxl protein. When the gonads of these pseudomales were analyzed for the *Sxl* splicing pattern, most of the RNA appeared to be spliced in the female mode. However, significant amounts of male spliced *Sxl* RNA could also be detected (Figure 4C). The gradual reduction in *Sxl* antibody staining intensity in more distal regions of the gonad suggests that this is likely to result from a failure in autoregulation as these undifferentiated cells age (see Figure 4A).

While the female-specific activation of *Sxl* does not appear to be affected by any of the *tra* mutant combinations, this is not the case for *orb.* As shown in Figure 4D, RNA expressed from the male promoter can be detected in gonads from tra^{v1}/Df and tra^1/Df *XX* animals. For tra^{v1}/Df about 10–15% of *orb* RNA is expressed in the male mode. A curious result was seen for the tra^1/Df genotype. Depending on the source of the tra^1 allele (Bloomington Stock Center or J. BELOTE), we observed either about 10% or about 40% male *orb* RNA. Since the genotype with respect to *tra* is the same, this difference in relative amounts of male and female *orb* RNA is presumably due to genetic background.

Despite this variability, these results show that an *XX* germline can express germline genes in the female mode in the complete absence of *tra.* This suggests that while *tra* may contribute to or augment the feminization of the germline it can not be the sole source of the somatic feminizing activity that is *tra2*-dependent.

Sxl: The findings described above indicate that null mutations in *tra* have only a small effect on the female-specific expression of *Sxl* and *orb* in the germline. Since mutations in *tra2* can completely block the female-specific expression of these germline markers, this would imply that there must be some other gene that utilizes *tra2* as a co-factor to effect the feminizing signal from the soma. This inference is consistent with our analysis of *orb* expression in the gonads of $Sxl^{M1,fm3}/Sxl^{fm7,M1}$ chromosomal females. Critical for our analysis, this mutant combination differentially affects *Sxl* function in the soma. Approximately 50% of the transheterozygous females survive so that this combination of *Sxl* alleles appears to retain at least some ability to control the dosage compensation pathway. On the other hand, this allele combination is apparently unable to regulate *tra* splicing (NAGOSHI *et al.* 1988) and $Sxl^{M1,fm3}/Sxl^{fm7,M1}$ animals have a very male-like morphology, including their body size. In spite of the fact that this genotype severely disrupts female *tra* regulation, it has no apparent effect on the feminization of the germline marker gene *orb* (Figure 5B). In addition, female-specific expression of *Sxl* is activated in the germline (Figure 5, A and B). These findings would also suggest that *tra* is not essential to send a feminizing signal to the germline.

Germline sexual identity is set in the embryo: The

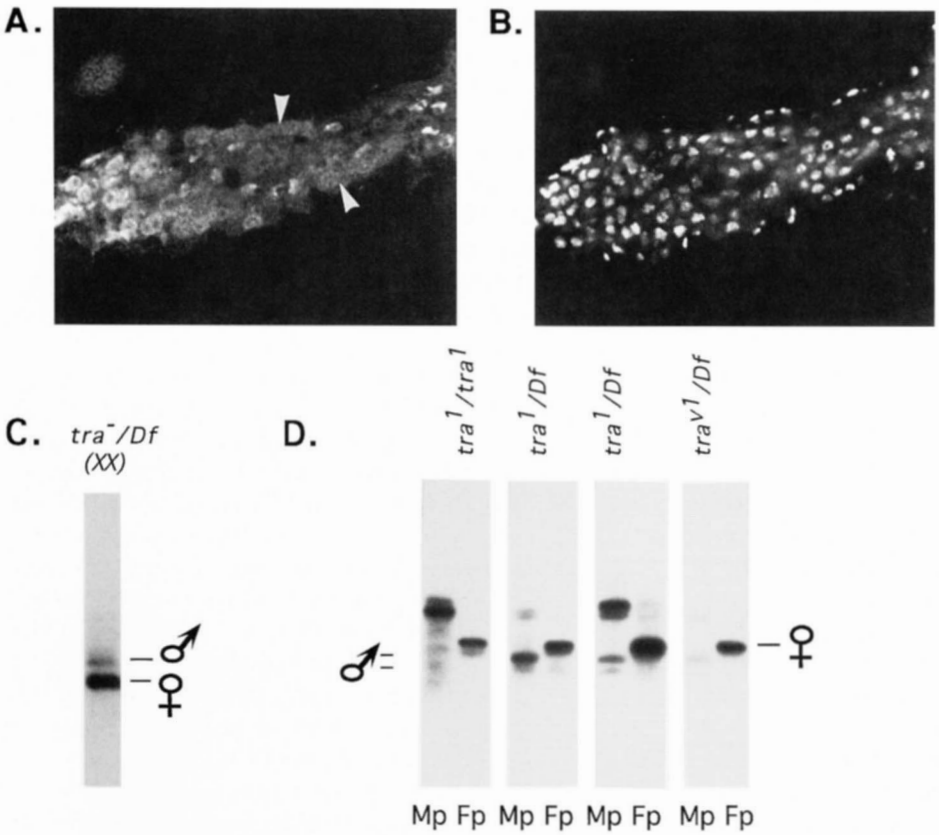

FIGURE 4.—Effect of altering *tra* activity on the germline expression of *Sxl* and *orb* in females. (A) Sxl protein staining in tra^1/Df pseudotestis. Note the strong cytoplasmic staining at the apical end of the pseudotestis, that appears to lessen towards the distal end (arrowheads). (B) Nuclear staining pattern of A. Both images were taken on a confocal microscope. Magnification was ×60. (C) *Sxl* RNA splicing pattern of tra^1/Df pseudotestis. A substantial amount of male spliced RNA is detected. (D) Expression of *orb* in females deficient for *tra* activity. The different genotypes are indicated. The tra^1/Df condition was generated from two separate tra^1 stocks (see text). Phenotypically all genotypes gave males. The bands above the expected male products are from genomic DNA. Symbols as for Figure 1.

results described above indicate that a *tra2*-dependent somatic signaling pathway is required for the female-specific expression of *Sxl* and *orb* in the germline. To better understand how the *Sxl-orb* pathway functions it is important to ascertain when in development the *tra2*-dependent feminization signal is first communicated to the germline. Unfortunately, we do not have the tools required to answer this question with a great deal of precision. The only available molecular marker for germline sex early in development is the *Sxl* gene; however, it is unlikely to be the first sex-specific gene expressed in *XX* germ cells, and its activation need not correspond to the time when the *tra2*-dependent feminization signal is first communicated. On the other hand, the timing of *Sxl* activation can be used to place an upper limit on the stage in development when this germline sex determination pathway must first operate.

Since morphological differences between male and female gonads are already evident by the first instar larva (KERKIS 1931), the pathway, and *Sxl* expression, should be initiated at an earlier stage—presumably at some point during embryogenesis. Hence, we examined *Sxl* expression in the germ cells of wild-type embryos at different stages of development. To identify the germ cells in the small embryonic gonads, we counter stained with *vasa* antibodies. As noted above, activation of *Sxl* in the germline is uncoupled from the soma and in early embryos the progenitor cells of the germline, the pole cells, do not express Sxl protein (BOPP *et al.* 1991). Moreover, the pole cells lack Sxl protein for as long as they are visible on the exterior of the embryo. Staining of older embryos indicates that *Sxl* remains off in female germ cells well beyond the time that pole cells are internalized and the gonad coalesces. *Sxl* antibody staining in *XX* germ cells can first be reliably detected in 16–20 hr embryo collections. At this time, female embryos (*i.e.*, those that express Sxl protein in the soma) had Sxl protein in their germ cells (as judged by *vasa* staining; see embryo in Figure 6). By contrast, none of the male embryos (*i.e.*, those lacking Sxl protein in somatic cells) at this developmental stage had Sxl protein-positive germ cells. As was the case in the larval gonad (see BOPP *et al.* 1993), Sxl protein in the *XX* embryonic germ cells is predominantly cytoplasmic. These results indicate that germline sexual development is initiated at least as early as 16–20 hrs of embryogenesis.

Time frame of the signal: If the regulatory strategies used in the *Sxl-orb* germline sex determination pathway are analogous to those employed in the soma, then the *tra2*-dependent signal should be required only transiently at the time sexual identity is initially selected. Thereafter, germline sexual identity should be controlled by a maintenance mechanism, that functions autonomously in the germ cells independently of the

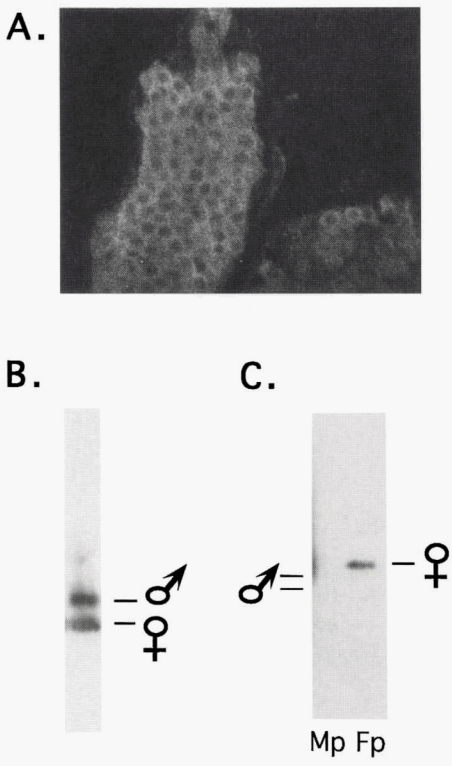

FIGURE 5.—Germline expression of *Sxl* and *orb* in $Sxl^{M1,fm3}/Sxl^{fm7,M1}$ females. (A) Confocal image of Sxl protein expression. Both pseudotestis in the image show Sxl protein. The protein distribution is cytoplasmic and some germ cells show weak to no Sxl protein expression. Magnification was ×60. (B) *Sxl* splicing in the pseudotestis. A large amount of male spliced RNA is detected. (C) *orb* expression is only in the female mode.

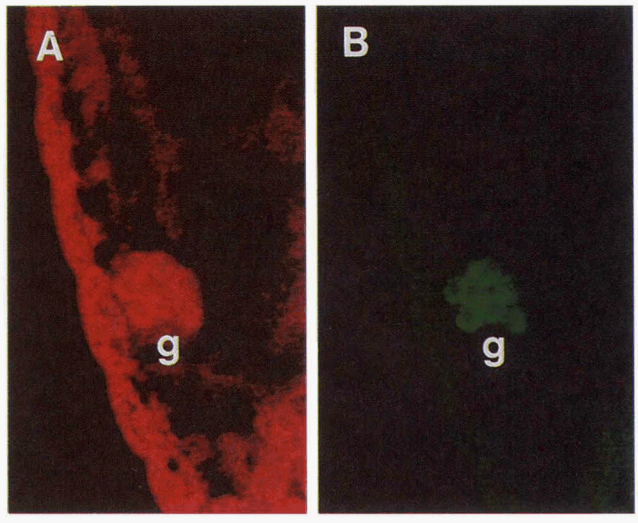

FIGURE 6.—Activation of Sxl protein in the embryonic gonad. Confocal images of an embryo from a 14–18-hr collection stained with anti-Sxl protein antibodies (A) and detected by Cy3-Streptavidin and anti-vasa protein antibodies (B) and detected by Fluorescein-Streptavidin. To observe the cytoplasmic staining in the germ cells the *Sxl* panel was underexposed. All tissues in this embryo were positive for Sxl protein. Staining with anti-vasa antibodies confirms the identity of the gonad (g). Magnification was ×60.

initiating signal, perhaps much like the *Sxl* autoregulatory feedback loop.

If this model for initiation and maintenance is correct, then the *tra2*-dependent somatic signal should become dispensable either just before or around the time when we first detect Sxl protein in female germ cells. To test this model we took advantage of the temperature sensitive *tra2* allele. $tra2^{ts2}/tra2$ embryos were collected and incubated for different times at the permissive temperature of 18° and then shifted to the nonpermissive temperature of 29° until the adult stage. Control animals were raised continuously at either the permissive or nonpermissive temperature. We then examined the pattern of *orb* expression in the germline of the resulting *XX* adults. (Only *orb* was assayed because the autoregulatory activity of *Sxl* would complicate interpretation of the temperature shift data.) We expected to find that $tra2^{ts}$ embryos would fail to properly signal female identity when shifted to the nonpermissive temperature prior to pathway initiation, *i.e.,* roughly midway through embryogenesis. Indeed, as shown in Figure 7 this expectation is correct; *orb* transcripts are expressed in the "male mode" when the temperature shift occurs prior to the time that we first detect *Sxl* protein in the germline. A different result should be obtained when the temperature shift occurs after pathway initiation; the loss of *tra2* activity should have no effect on sexual identity and *orb* should be expressed in the "female mode." Surprisingly this is not the case; *orb* transcripts are expressed in the "male mode" not only in 24-hr embryos but also through the first and into the second instar larval stage. In fact, the *XX* $tra2^{ts}$ animals remain sensitive to the temperature shift until midway through the third instar larval stage.

These findings are inconsistent with the model of a transient germline sex determination signal. They suggest that the *tra2*-dependent signaling system is required continuously, at least until the mid-third instar larval stage, to faithfully maintain a commitment to the female pathway. If this is correct, then the soma must be capable of signaling and the germline capable of responding to the signal as late as this larval stage. To test this, $tra2^{ts}$ embryos were collected at 29° and then shifted to 18° at different times. As can be seen in Figure 7, the *tra2*-dependent signal can initiate female-specific *orb* expression in *XX* germ cells not only in larval stages, but as late as the early pupal stage.

DISCUSSION

***Sxl* is not the master regulatory gene in the germline:** The results presented here, together with the recent studies of BAE *et al.* (1994) indicate that the *Sxl* gene can not function as the master regulatory switch in germline sex determination. Two lines of evidence

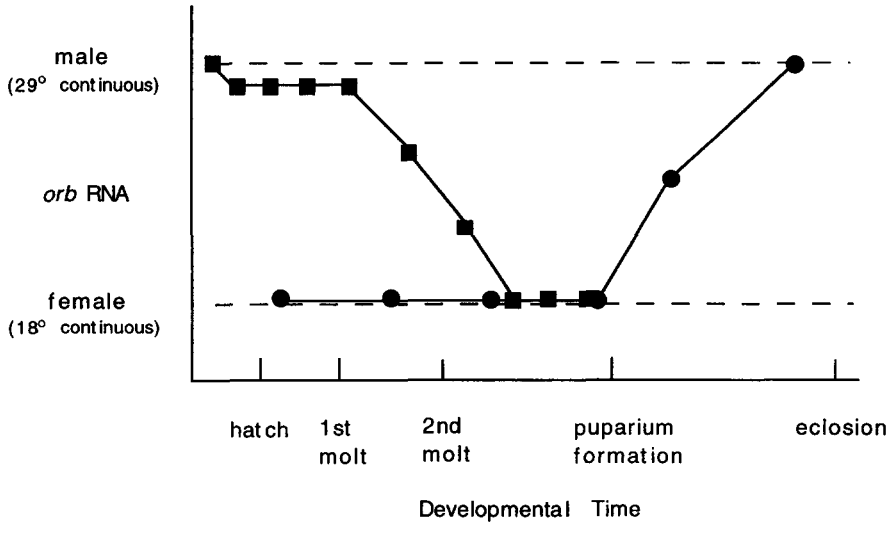

FIGURE 7.—Effect of temperature up and down shifts during development on the sex-specific expression of *orb* in $tra2^{ts2}/tra2$ *XX* animals. Embryos at 18° or 29° were shifted to 29° or 18°, respectively, at varying times during development. The expression of *orb* was monitored in the resulting adults. (●) marks the downshift series after 24, 48, 72, 96, 120 and 168 hr at 29° while (■) marks the upshift series after 12, 24, 48, 72, 96, 120, 168, 192, 216 and 240 hr at 18°. When continuously at 18° *orb* is in the female mode only; at 29° *orb* is in the male mode. The proportion of male *vs.* female *orb* RNA at each time point is shown by the relative position between the all male *vs.* all female expression lines.

support this assertion. The first comes from an analysis of germline sexual identity in mutant backgrounds in which *Sxl* is not properly autoregulated and *XX* germ cells in the adult female gonad have no detectable Sxl protein. snf^{fs1621} is a mutation in a gene encoding a generic splicing factor associated with U1 snRNP (FLICKINGER and SALZ 1994), while otu^{1} is a mutation in a gene encoding a germline-specific cytoplasmic protein of unknown function (STEINHAUER and KALFAYAN 1992). Both mutations have sex-specific effects in the germline; when homozygous in females, they cause an early arrest of oogenesis but they have no apparent effect on gametogenesis in males. The early oogenic arrest in snf^{fs1621} is likely to be the direct consequence of a failure in *Sxl* autoregulation (and the lack of Sxl protein) since gain-of-function mutations in *Sxl*, such as $Sxl^{M\#1}$, which constitutively express Sxl protein suppress the oogenesis defect. In contrast, otu^{1} appears to have more pleiotrophic effects, and the defects in oogenesis in this mutant appear to involve processes beyond simply failing to produce Sxl protein. In spite of the fact that *XX* germ cells homozygous for either mutation do not express detectable amounts of Sxl protein, the sexual identity of the mutant germ cells, as judged by the pattern of *orb* expression, is female not male (see also BAE *et al.* 1994).

The second line of evidence comes from the reverse condition which occurs in the germline of $tra2^{ts2}/tra2$ *XX* animals grown under nonpermissive conditions. Even though Sxl protein is present in these germ cells, *orb* is expressed in the male not the female mode. Thus, *Sxl* expression is not in itself sufficient to induce feminization of the germ cells. Consistent with the view that *Sxl* does not function as the master switch of sexual identity in the germline, the constitutively active *Sxl* mutations, $Sxl^{M\#1}$ and $Sxl^{M\#4}$, do not feminize *XY* pole cells but allow normal gametogenesis in a male soma (STEINMANN-ZWICKY *et al.* 1989; STEINMANN-ZWICKY 1993). Similarly, when Sxl protein is ectopically expressed in the male germline from a cDNA construct, it does not impose female development and the transgenic males are fertile (D. BOPP, unpublished data).

If *Sxl* is not the master switch in germline sex determination, why is it required in *XX* germ cells for normal oogenesis? It could function in one of several subordinate germline sex differentiation pathways, perhaps eliciting the female-specific expression of a small group of target genes. Consistent with this possibility is the fact that the expression of several sex-specific germline enhancer trap lines and some genes (*e.g., Stellate*) appears to depend upon *Sxl* (see WEI *et al.* 1994). An alternative view is that the functions of *Sxl* during oogenesis are not at all related to the system controlling germline sexual identity but rather *Sxl* is required for the proper elaboration of developmental processes that are unique to oogenesis much like other genes, such as *bam, fu, orb, egal, Bic-D*, etc., that function in the differentiation of the female germline. This view would be consistent with the germline phenotypes observed in genetic backgrounds deficient in *Sxl* activity (SCHÜPBACH 1985; BOPP *et al.* 1993).

Model for sex determination in the germline: How is sex chosen and remembered in the germline? Pole cell transplantation experiments have suggested that autonomous and nonautonomous components are important in germline sex determination. We will discuss the nonautonomous component, the somatic communication pathway, first as our findings bear most directly on its role in the sex determination process.

Neither dsx nor tra are essential for the somatic communication pathway that controls Sxl and orb expression in the germline: Both OLIVER *et al.* (1993) and STEINMANN-ZWICKY (1994a,b) have argued that the somatic signal for female sexual identity in the germline is mediated by the *tra* → *dsx* somatic sexual differentiation pathway. In the

former case, this conclusion was based on the detection of male *Sxl* RNA in adult gonads of *tra* and *dsx* mutants, while in that latter, it was based (in part) on the altered size of the gonad in mutant larvae. Our results are most clear cut for *dsx*. Like OLIVER *et al.* (1993), we have found low levels of male-spliced *Sxl* RNA in gonads of various *dsx* mutant combinations. However, this male RNA does not appear to arise from a failure in initiating female-specific expression of *Sxl* in the germline. In fact, high levels of Sxl protein are evident, particularly in germ cells at the apical end of the mutant gonads. A more likely explanation is that the male *Sxl* RNA arises from an occasional failure in *Sxl* autoregulation as the germ cells proceed down the gonad and attempt to differentiate. Similar failures in *Sxl* autoregulation are evident in other mutants such as *bam* or *fu*, that perturb the early steps in the formation of a 16-cell cyst and disrupt the redistribution of cytoplasmic Sxl protein. These observations indicate that *dsx* is not required to activate *Sxl* expression in the female mode in *XX* germ cells. That *dsx* is not essential for the feminization of *XX* germ cells is supported by our analysis of *orb* expression. We found that *orb* is expressed in the female mode in *XX* germ cells not only in the absence of *dsx* activity, but also when *dsx* is exclusively male. In addition, since we find that *orb* is in the male mode in the germ cells of dsx^- *XY* males, it also appears that *dsx* activity is not required for the masculinization of *XY* germ cells.

While *dsx* does not seem to play a role in the *Sxl-orb* germline sex determination pathway, it is clearly required in the female soma for normal oogenesis and fertility. The phenotypic effects of various *dsx* mutant combinations on gonadal development may be instructive in this regard. The least severe disruptions in oogenic differentiation are evident under conditions where both the male and female forms of the dsx protein are expressed (dsx^+/dsx^D). In these gonads we observe nearly normal looking egg chambers consisting of an oocyte at the posterior, nurse cells at the anterior, and a surrounding array of somatic follicle cells. However, some aspects of oocyte-nurse polarity seem to be perturbed since we observe high levels of Sxl protein in the oocyte. This is presumably a consequence of a failure in some aspect of germline-soma communication. In addition, vitellogenesis which normally initiates around stage 7 or 8 does not occur. In the absence of *dsx* activity (dsx^-/dsx^-), the germ cells also initiate oogenic differentiation and appear to form 16 cell cysts; however, these egg chambers do not develop properly. Though polyploid nurse cells are present, we can not detect an oocyte at the posterior end, and somatic cells do not properly envelop the cyst. This finding would suggest that the repositioning of the oocyte to the posterior of the 16-cell cyst may require a *dsx*-dependent signal from soma to germline (see also LANTZ et al. 1994). Finally, in a soma which expresses only the male dsx protein (dsx^D/Df) there is no apparent oogenic differentiation, and only small undifferentiated germ cells are observed. This finding would indicate that in a soma expressing only the male form of the dsx protein, the germline cystoblasts are unable to properly execute the pathway that ultimately generates a 16-cell cyst (see Figure 8).

The malfunctioning of the somatic cells in the adult dsx^- gonad may be relevant to the findings of STEINMANN-ZWICKY (1994a) that the gonads of dsx^- female larvae are more male-like in size and the gonads of dsx^- male larvae are more female-like in size. Gonadal size is related to germ cell proliferation and, if both males and females modulate the rate of germ cell proliferation through *dsx*, then this signaling process would be disrupted in both sexes by the absence of *dsx* activity.

As might be expected from the fact that *dsx* is expressed in the male mode in the absence of *tra* function, the gonad of tra^- females resembles that observed in dsx^D/Df females. There is no evidence of oogenic differentiation and the male-like somatic gonad is populated by clusters of small undifferentiated germ cells. Like OLIVER *et al.* (1993) we detect male-spliced *Sxl* RNA in the tra^- mutant gonads; however, the pattern of Sxl protein accumulation in these germ cells again indicates that this is probably due to an occasional failure in autoregulation, rather than a failure in germline sex determination. The presence of *predominantly* female *orb* RNA in genetic backgrounds null for *tra* function would also indicate that *tra* is not essential for communicating "female identity" to the germline. While *tra* does not appear to be *essential* for somatic signaling of germline sexual identity, we can not exclude the possibility that *tra* may enhance this communication or may have a partially redundant function in the communication pathway. This ambiguity about the role of *tra* comes from the fact that we detect some male *orb* RNA in two of three *tra* null mutant backgrounds. The relationship of *tra* to the somatic communication pathway will be discussed further below.

The somatic communication pathway requires tra2: Like *tra* and *dsx*, *tra2* is required in the female soma, but not the germline, for normal oogenesis. However, while neither *tra* or *dsx* seem to play a critical role in signaling the feminization of *Sxl* and *orb*, *tra2* is absolutely essential; in the absence of *tra2* function, both these marker genes are expressed in the male not the female mode. Since *tra2* is expressed constitutively in both sexes, it is unlikely that a somatic feminization signal could originate from *tra2*. Rather, one must suppose that as in the *tra* → *dsx* sexual differentiation pathway, *tra2* functions as a co-factor for some other sex-specific gene, "*X*." This is shown in the model diagrammed in Figure 8. In this model we have placed *Sxl* upstream of *X*, and it

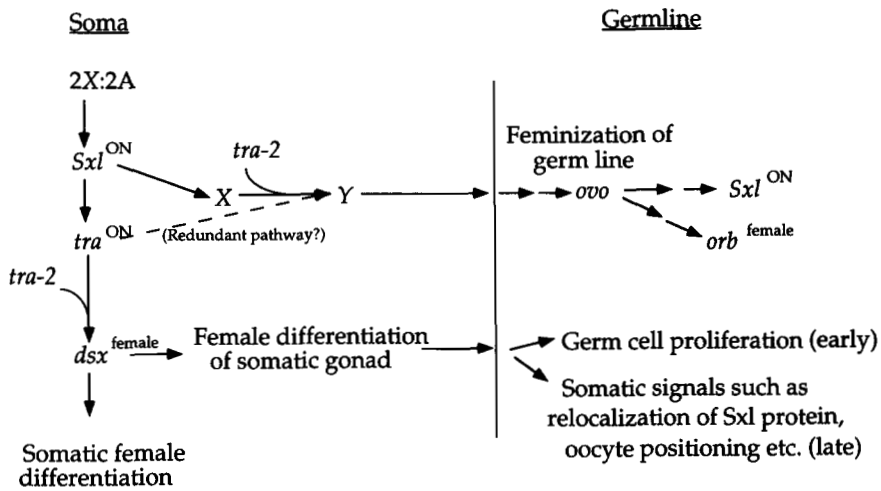

FIGURE 8.—Model of soma to germline signaling. The somatic sex determination pathway is shown on the left. Arrows depict signals sent to the germline by the soma with the vertical line representing the boundary between the two. The signal that sets in motion female-specific gene expression in the germline is considered the germline sex determination signal. As *tra* is not essential for the female expression of *Sxl* or *orb* in the germline, its contribution to germline feminization is shown as being redundant. The unknown genes have been labeled *X*, *Y* and *Z*. While *ovo* is a good candidate for *Z* (see text), there could be several intervening genes between *ovo* and *Sxl* and *ovo* and *orb*, hence the multiple arrows. Signals from the soma that are necessary for correct differentiation of the somatic component of the germline, germ cell proliferation (STEINMANN-ZWICKY 1994a) and for elaboration of germline differentiation that are *tra*- and *dsx*-dependent are also shown. While the *tra* → *dsx* pathway is not required for setting the sex-specific expression of *Sxl* and *orb* in germ cells, we can not exclude the possibility that the *tra* → *dsx* signaling pathway may direct proper oogenesis by "feminizing" the expression of some as yet unknown genes in the germline.

activates the somatic signaling pathway by directing the female-specific expression of *X*. (Though it seems reasonable to assume that *Sxl* regulates *X*, it should be emphasized that we have no direct evidence for such a somatic *Sxl* function.) The female X protein, together with tra2 protein, would then regulate the activity of a downstream target gene, "*Y*." We presume that the regulation of *Y* is post-transcriptional and involves interactions between X and tra2 that are quite similar to those between tra and tra2 proteins in the regulation of *dsx*. If tra and X proteins interact with the same domain(s) in the tra2 protein, it is possible that these two genes are partially redundant in the somatic communication pathway. A redundancy of this sort could explain the weak and variable effects of *tra* mutants on the sexual state of the *orb* gene. It would also be consistent with the observation that ectopic expression of tra protein from a constitutive *hsp83:tra* (female) cDNA transgene can partially feminize the germline of chromosomal males, activating both *Sxl* and *orb* to the female mode. This feminization of the male germline appears to be a consequence of somatic *tra* expression from the transgene and, like the female signal described here, requires the activity of *tra2* (J. I. HORABIN, D. BOPP, J. WATERBURY and P. Schedl, unpublished observations; see also NAGOSHI et al. 1995).

Since the default state of the signaling pathway appears to be male, we presume that when the female form of *Y* is expressed it functions to send a feminization signal from the soma to the germline (and not to block the expression of a masculinizing signal). If *Y* is similar to *dsx*, which is a transcription factor, there may be several steps between expression of the female Y protein and the actual signal to the germline.

What is the target for the somatic signaling system? The results presented here and in BAE *et al.* (1994) indicate that *Sxl* can not be the key target of the somatic signaling system. Some other gene (or genes), "*Z*" in Figure 8, is activated when germ cells receive this signal and it directs the female-specific expression of downstream targets such as *Sxl* and *orb*. From pole cell transplantation experiments, it is clear that *XX* germ cells are much more capable of responding to the feminization signal than are *XY* germ cells (STEINMANN-ZWICKY 1994a). This could be due to the operation of an autonomous *X*/A counting system in germ cells that is analogous to (but different from) that found in the soma. Alternatively, it may simply reflect the dose effects of one or several *X*-linked genes that are the target(s) for the somatic signal. In this view, the presence of two copies of the *X*-linked genes would enhance the response to the somatic feminization signal. In either case, a good candidate for gene *Z*, the germline target of the somatic feminization signal, is *ovo*. Expression of *ovo* in the germline appears to be dependent on the *X* chromosome dose (OLIVER *et al.* 1994) and null mutations have been reported to cause the early death of female germ cells (OLIVER *et al.* 1987, 1994).

Initiation and maintenance: Our results indicate that the regulatory strategies used for initiation and mainte-

nance in the *Sxl-orb* germline sex determination pathway are quite different from those employed in the soma. In the soma, an autonomous and transient signal, the *X*/A ratio, is used in the initial choice of female sexual identity early in embryogenesis. This signal sets in motion an autoregulatory feedback loop which then serves to maintain the determined state in each cell for the rest of the life cycle. The choice of sexual identity in the *Sxl-orb* germline pathway also depends upon a signal. However, the signal is not autonomous but appears to require the soma to inform the germline of its appropriate sexual identity. Additionally, the somatic signal does not function transiently. Instead, it is required over an extended period of time to commit the germ cells to the female state and thus appears to function not only in the setting but also in the memory of sexual identity. Of course, if the activity of the somatic signaling pathway is controlled by *Sxl* (as diagrammed in Figure 8), it is the *Sxl* autoregulatory feedback loop in the soma that ultimately functions to initiate and maintain female identity in the germline.

In our temperature-shift experiments, the timing of the signal defined by up-shifts and down-shifts did not coincide. The former indicated that the somatic signal is required through the mid-third instar larval stage, while the latter showed that the signaling system can feminize the germline as late as the pupal stage. Whether this discrepancy is meaningful is not clear. In the up-shift experiment, it is conceivable that the somatic signal may continue to function for some time after the shift to the nonpermissive temperature. For instance, it may take several days for active tra2 protein to decay after the up-shift (*cf.* BELOTE *et al.* 1985). Additionally, the downstream proteins in the pathway (*e.g.*, Y) may not turnover immediately after the loss of *tra2* activity. If this is the case, then the upshifts will underestimate the latest time in development when the signaling pathway is functional. This would suggest that the somatic signal may not only function but may also be required into the pupal stage.

From the time the *Sxl-orb* germline sex-determination pathway is first activated midway through embryogenesis until perhaps as late as the pupal stage, the germline appears to be incapable of autonomously remembering its sexual identity. The obvious question then is why does the somatic signal become dispensable? While it is plausible that an autonomous memory system becomes activated in the germline at or just before the pupal stage, an alternative, and equally attractive possibility, is that the end of the signaling period marks the formation of oogenic stem cells which irreversibly commits the fate of germ cells to the "female" pathway—oogenesis. If this is the case, there may be no key sex *determination* gene (like *Sxl* in the soma) in germ cells that controls all aspects of female germline development. Instead, the soma may function as the "master regulatory switch" for the germline, continuously transmitting cues which activate different female-specific differentiation pathways.

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